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Electron Microscope Study of a Plasmid Chimera Containing the Replication Region of the *Escherichia coli* F Plasmid[1]

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pML31, a plasmid chimera constructed to contain the replication genes of an F*lac* plasmid, has been studied by electron microscope methods. Heteroduplex analysis shows that the only F sequence present in pML31 is that with coordinates 40.3-49.3F. This region has previously been identified as essential for plasmid maintenance. The sequence of pML31, which was derived originally from R6-5, carries the *km* gene(s) and an inverted duplication of a 1.0-kilobase sequence. On the basis of length measurements, the repeated sequence is different from IS1, IS2, IS3, and an inverted repeat associated with the *km* gene(s) of plasmid JR67.

The use of restriction endonucleases (25) has opened a new approach to the study of large deoxyribonucleic acid (DNA) genomes. Use of these enzymes, which make double-stranded breaks at specific short deoxynucleotide sequences, makes possible the isolation of specific fragments from long DNA sequences. Such small fragments contain only a limited amount of genetic information, enough to code for a small number of functions. These fragments can be used for the analysis of the functions in the absence of a background of a large and unwieldy amount of DNA that does not carry any information pertaining to the functions of interest. As an example of the use of such techniques, two groups (23, 38) have each recently isolated a fragment from an F*lac* plasmid that contains genetic information for the plasmid maintenance (replication and segregation) system(s).

Lovett and Helinski (23) mixed an *Eco*RI digest of an F*lac* plasmid with a purified *Eco*RI fragment obtained from the plasmid chimera pML21 (but originally derived from plasmid R6-5). The fragment derived from pML21 cannot replicate autonomously but does carry genes that determine resistance to the antibiotic kanamycin. After ligation of the mixture and transformation of a kanamycin-sensitive recipient, Lovett and Helinski isolated a kanamycin-resistant transformant that harbored an autonomously replicating R plasmid (pML31). pML31 is comprised of the *km* fragment of pML21 and a single *Eco*RI fragment of F*lac*. Since the *km* fragment cannot replicate autonomously, at least some of the information that allows pML31 to exist in the extrachromosomal state must be provided by the F*lac* fragment. We present here an electron microscope study of pML31 in which we have mapped the location, on the genetic map of F, of the fragment derived from F*lac*. We have also found that the *km* fragment contains an inverted duplication that appears to be different from the inverted duplications found in other R plasmid systems.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used are shown in Table 1.

Isolation of DNA. Cultures were grown as described previously (18, 36). pML31 was isolated from a cleared lysate (18). A final Triton concentration of 0.1% was used. F8-33 and FΔ(33-43) were isolated from Sarkosyl lysates (36). Plasmid DNA was purified by buoyant density centrifugation in CsCl in the presence of ethidium bromide (27, 36). Supercoiled plasmid DNA was X-ray nicked (36). After recentrifugation in CsCl-ethidium bromide, the upper DNA band was used for heteroduplex experiments.

Preparation of heteroduplexes and electron microscopy. DNA was mixed, denatured, renatured, and mounted for electron microscopy by the formamide technique described previously (27, 36). A Phillips 300 electron microscope was used. Micrographs were traced on overhead projections using a Hewlett-Packard 9280A calculator and a Hewlett-Packard 9864A Digitizer.

RESULTS

Characterization of pML31. The molecular length of singly nicked, double-stranded pML31

was determined relative to a ϕX174 RFII standard. pML31 was found to be 3.18 times the size of ϕX174, or 16.2 kilobases (kb) (Table 2). This agrees well with the 10.5×10^6-dalton estimate obtained from sedimentation data (23).

Single strands of pML31 DNA were examined after denaturation and a short (20 min) incubation to permit intrastrand renaturation. Many of the molecules (45 out of 50 counted) assumed a dumbbell configuration in this experiment (Fig. 1). Such dumbbell molecules contain a short double-stranded region (1.0 ± 0.1 kb; Table 2) bounded by a single-stranded loop at each end; the single-stranded loops are of unequal sizes (1.0 ± 0.2 and 12.9 ± 0.7 kb, respectively; Table 2). The dumbbell configuration indicates that pML31 contains an inverted duplication of a 1-kb sequence. As will be shown below, the inverted repetition is located in a region of the molecule that does not hybridize to F sequences; thus we conclude that the inverted repeat is carried in the *Eco*RI fragment derived from pML21.

pML31/F8-33 heteroduplex. F8-33 is an F′ plasmid which carries an inverted duplication of the IS2 sequence (36). The loop formed by intramolecular renaturation of the repeated IS2 sequence provides a useful marker for heteroduplex studies (Fig. 2a). The coordinate system used to located sequences on F′ plasmids has been described in detail in previous publications (14, 27); it is briefly explained in the legend to Fig. 2a. The defined origin and terminus of the circular map is 94.5/0F. Coordinates in kilobase units of F sequences relative to this origin are indicated by the suffix F. Coordinates of bacterial chromosomal sequences are denoted by the suffix B. Figure 2a shows that the chromosomal sequences of F8-33 have a length of 22.0 kb. The two junctions of F DNA

TABLE 1. *Bacterial strains*

Strain	Plasmid	Chromosomal genotype	Reference
JE3513	F8-33	*thr⁻ leu⁻ gal₂⁻ lac⁻ pil⁻ fla⁻ str⁻*	36
W1655	FΔ(33-43)	*metB⁻λ⁻λʳ*	1
CR34(pML31)	pML31	*thr⁻ leu⁻ thi⁻ lac⁻ thy⁻*	M. Lovett[a]
CSH2(R6)	R6	Prototrophic	15

[a] Constructed by transformation of CR34 with pML31 DNA.

TABLE 2. *Molecular lengths of various DNA segments*

Feature	Molecular length (kb)[a]	No. of measurements
Native circular pML31	16.2 ± 0.5	30
Single-stranded circular pML31		
Double-stranded stem	1.0 ± 0.1	20
Small single-stranded loop	1.0 ± 0.2	20
Large single-stranded loop	12.9 ± 0.7	19
pML31/F8-33		
Large duplex region(cd)[b]	9.0 ± 0.2	19
Single-stranded region between IS2 loop and duplex region(bc)	5.1 ± 0.3	11
Shorter single-stranded region between duplex and inverted repeat(de)	1.5 ± 0.1	14
Longer single-stranded region between duplex and inverted repeat(ce)	2.3 ± 0.2	18
Inverted repeat stem(ef)	1.0 ± 0.1	20
Inverted repeat loop	1.0 ± 0.1	18
pML31/FΔ(33-43)		
Large duplex region(cd)	6.4 ± 0.3	15
Shorter single-stranded region between duplex and inverted repeat(de)	1.6 ± 0.2	15
Longer single-stranded region between duplex and inverted repeat(ce)	4.6 ± 0.4	14
Inverted repeat stem(ef)	1.0 ± 0.1	19
Inverted repeat loop	1.0 ± 0.1	19

[a] The lengths of double- and single-stranded features were measured relative to ϕX174 RFII and ϕX174 single-stranded circles, respectively. The relative measurements were converted to absolute lengths by taking the size of ϕX174 DNA to be 5.1 kb.

[b] The letters in parentheses refer to the letters used to identify the specific features of the heteroduplexes in Fig. 2b, 3, 4, and 5.

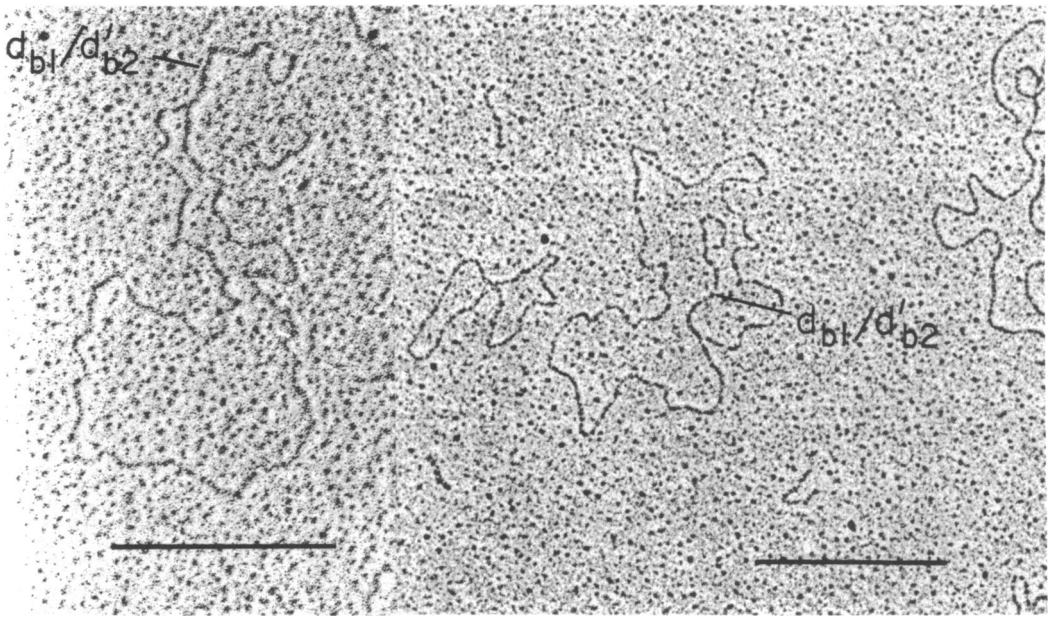

FIG. 1. *Electron micrographs of circular single strands of pML31 DNA, demonstrating the occurrence of an inverted duplication. The inverted duplication is labeled d_{b_1}/d'_{b_2} because it is identical with the d_{b_1}/d'_{b_2} inverted repeat of R6-5 (15, 35); see footnote d of Table 3). Bar represents a length of 0.5 μm.*

with chromosomal DNA occur at 8.5F/6.5B and at 28.5B/16.3F. The inverted repeat of IS2 involves the sequence 16.3-17.6F paired with an IS2 sequence inserted in an inverted sense at 35.2F.

An electron micrograph of a pML31/F8-33 heteroduplex is shown in Fig. 3. The heteroduplex structure deduced from many such micrographs is diagrammed in Fig. 2b. F8-33 was found to hybridize to only a portion of pML31. The region of homology between F8-33 and pML31 is 9.0 ± 0.2 kb in size (Table 2), which agrees well with independent estimates of the size of the F *Eco*RI F fragment of pML31 (6×10^6 daltons; 23). All of the plasmid-coded replication functions that pML31 and F display in common must be coded for by this 9,000-base pair sequence.

The hybrid region in the pML31/F8-33 heteroduplex is located 5.1 ± 0.3 kb from the base of the IS2 inverted repeat of F8-33 (Table 2, Fig. 2b), which itself is located at 16.3/35.2F on the genetic map (Fig. 2a; 15; E. Ohtsubo and M. T. Hsu, personal communication). Because of the ambiguity involved in orienting a feature on a circular molecule relative to a single reference point, the replication region can have either of two locations on F8-33 according to this result: either 40.3-49.3F or 14.4-23.4B. We propose that the replication genes of F map in the region 40.3-49.3 for two reasons. Ohtsubo and Ohtsubo have mapped the coordinates of a 9-kb *Eco*RI fragment of F (fragment 5) as 40.4-49.4 (personal communication), which agrees well with the former alternative. Furthermore, it seems very unlikely that a sequence that contains the information for the replication functions of F would hybridize to chromosomal sequences of the F', as the second alternative requires.

pML31/FΔ(33-43) heteroduplex. The location of the hybrid region in pML31/F8-33 as 40.3-49.3F was confirmed by examination of heteroduplexes formed between pML31 and FΔ(33-43). FΔ(33-43) is a deletion mutant of F, which is missing the sequence 32.6-42.9F (1); it contains no chromosomal sequences. pML31/FΔ(33-43) heteroduplexes display a duplex region 6.4 ± 0.3 kb in length (Fig. 4, Table 2). The straightforward interpretation of the structure of this heteroduplex is shown in Fig. 5. The shorter of the two single-stranded regions located between the duplex region and the inverted repeat of pML31 is the same length in the pML31/FΔ(33-43) heteroduplex as it is in the pML31/F8-33 heteroduplex. This means that the nucleotide sequence of pML31 adjacent to this single-stranded region is homologous to both reference plasmids. Therefore, that region of pML31 cannot be homologous to chromo-

somal sequences of F8-33 but must be homologous to the F sequences 40.3-49.3F. Since 40.3-49.3F overlaps the 32.6-42.9F deletion of FΔ(33-43), it would be expected that the hybrid region of the pML31/FΔ(33-43) heteroduplex would be shorter than the hybrid region of the pML31/F8-33 heteroduplex by an amount of (42.9-40.3) or 2.6 kb. Similarly, the longer of the two single-stranded regions between the duplex region and the inverted repeat of pML31 should be 2.6 kb longer in the pML31/FΔ(33-43) heteroduplex than in the pML31/F8-33 heteroduplex. Our observations agree with these predictions, the duplex regions being 6.4 and 9.0 kb and the single-stranded regions being 4.6 and 2.3 kb in pML31/FΔ(33-43) and pML31/F8-33, respectively (Table 2).

The inverted duplication on pML31 is neither IS2 nor IS3. The sequence of pML31 that did not hybridize to F8-33 must be the sequence derived from pML21, since that fragment was ultimately derived from an *Eco*RI fragment of R6-5 that carries the *km* gene and is located in a region of the R6-5 molecule that is not homologous to F(35). This pML31 sequence contains an inverted repeat structure identical to that found in the nonhybridized pML31 single-stranded circles (Table 2). The presence of both this inverted repeat and the inverted duplication of IS2 of F8-33 in the same heteroduplex structure allows a direct comparison of the sizes of the double-stranded stems. The IS2 stem was found to be 1.3 ± 0.1 kb, which agrees well with previous estimates (7, 13, 15, 33). In comparison, the inverted repeat sequence of pML31 was 1.0 ± 0.1 kb (Table 3). The ratio of the sizes of these two repeats is therefore 0.80, indicating that the two sequences are of different sizes. To obtain more data, the lengths of the inverted repeats of all the molecules in the micrographs, not just those in heteroduplexes, were considered. The results show the same difference in

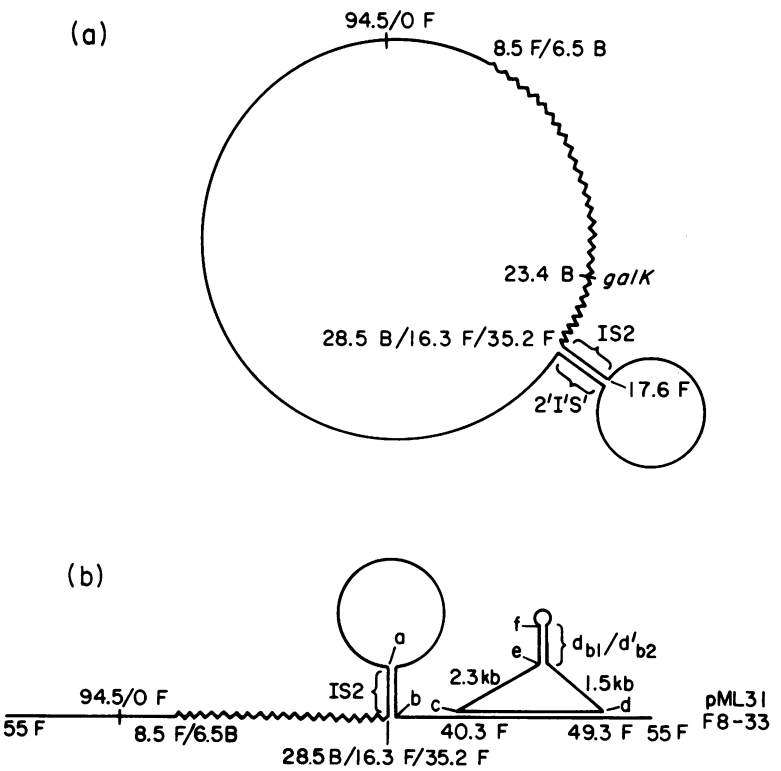

FIG. 2. *(a) Representation of the structure of F8-33. The system of map coordinates and the IS2 inverted repeat feature are described in the text. The coordinates of F8-33 have been recently revised (E. Ohtsubo and M.-T. Hsu, personal communication). The precise location of the galK cistron has been determined (E. Ohtsubo and M.-T. Hsu, manuscript in preparation). (b) A diagram (not to scale) of the pML31/F8-33 heteroduplex. For convenience in representation, the circular F8-33 strand is shown in linear form. The symbols a through f denote the same points on the heteroduplex as they do in Fig. 3. The system of map coordinates is described in the text.*

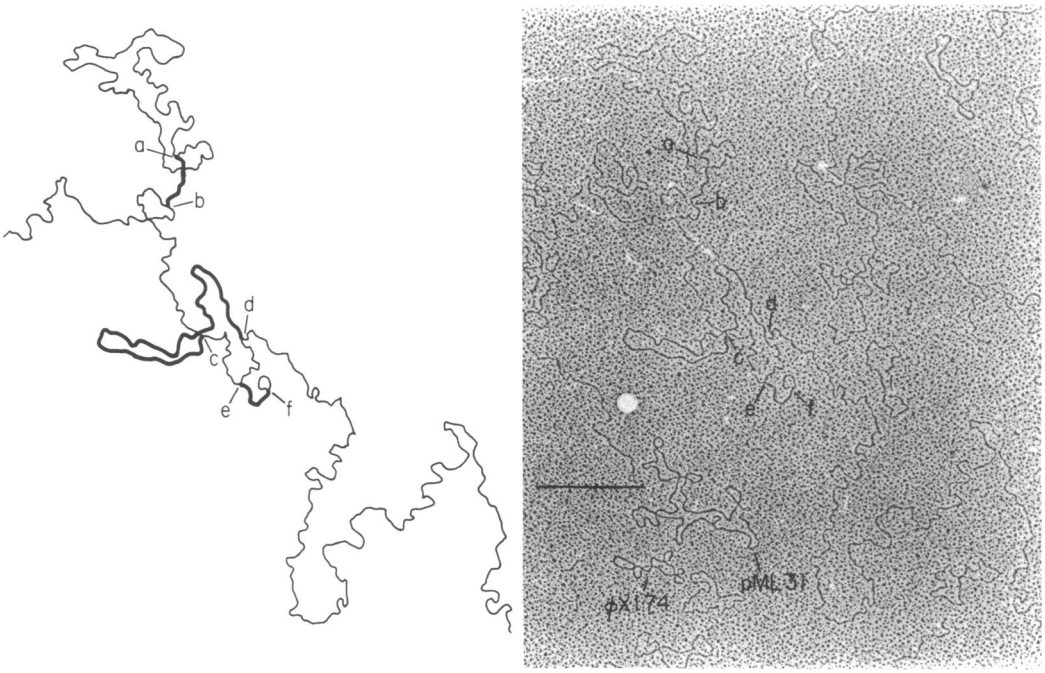

FIG. 3. *Electron micrograph of a heteroduplex between a circular single strand of pML31 and a broken single strand of F8-33. A tracing of the heteroduplex is also shown. Arrows a and b denote the ends of the inverted repeat of F8-33 used as the reference feature. Arrows c and d denote the ends of the region of homology between pML31 and F8-33. Arrows e and f denote the ends of the inverted repeat of pML31. Bar represents a length of 0.5 μm.*

the lengths of the two sequences (ratio = 0.80; Table 3).

The insertion sequence IS3 is the same length as IS2 (7, 16), so the repeated sequence of pML31 is not identical to the IS3 sequence. To demonstrate this directly, a mixture of pML31 and R6 (which contains an inverted duplication of IS3) was denatured, allowed to renature for 20 min, and spread. The stem of the inverted repeat of pML31 was again found to be 1.0 ± 0.1 kb, whereas the IS3 stem was found to be 1.3 ± 0.1 kb, as expected. Figure 6 shows a histogram of the measurements made of IS2 and the inverted repeat of pML31 and of IS3 and the pML31 duplication. These data clearly show that the two IS sequences are of different lengths than that of the pML31 sequence.

DISCUSSION

Our results have revealed interesting aspects about each of the two *Eco*RI fragments of the chimeric plasmid pML31. The larger of the two fragments (9.0 kb) carries genes that allow pML31 to be maintained autonomously. The maintenance of pML31 resembles that of the parent F*lac* in every aspect tested (copy number, stability, and incompatibility [23] and sensitivity to curing by acridine orange; W. Maas, personal communication). By these criteria, the F*lac*-derived *Eco*RI fragment of pML31 contains all of the genetic information present in F itself for the plasmid-determined components determining these aspects of the F maintenance system. These results do not, however, rule out the presence of information on F that is not contained in pML31, since other aspects of F maintenance (e.g., thermosensitivity of wild-type F in certain host strains [37, 39] or the ability to integratively suppress certain *dna* mutations [26, 39]) have not been tested with pML31.

The heteroduplex studies presented here demonstrate that the F*lac*-derived component of pML31 is homologous to the region on the map of F with coordinates 40.3-49.3F. Previous assessments of the location of the replication functions of F(6) were based on the properties of plasmids that were deleted for various regions of the F genome but retained the facility to replicate autonomously. From the map locations of such deletions, it was estimated that the maintenance genes on F mapped between 42.9 and 52.2F. Thus, the present results are in

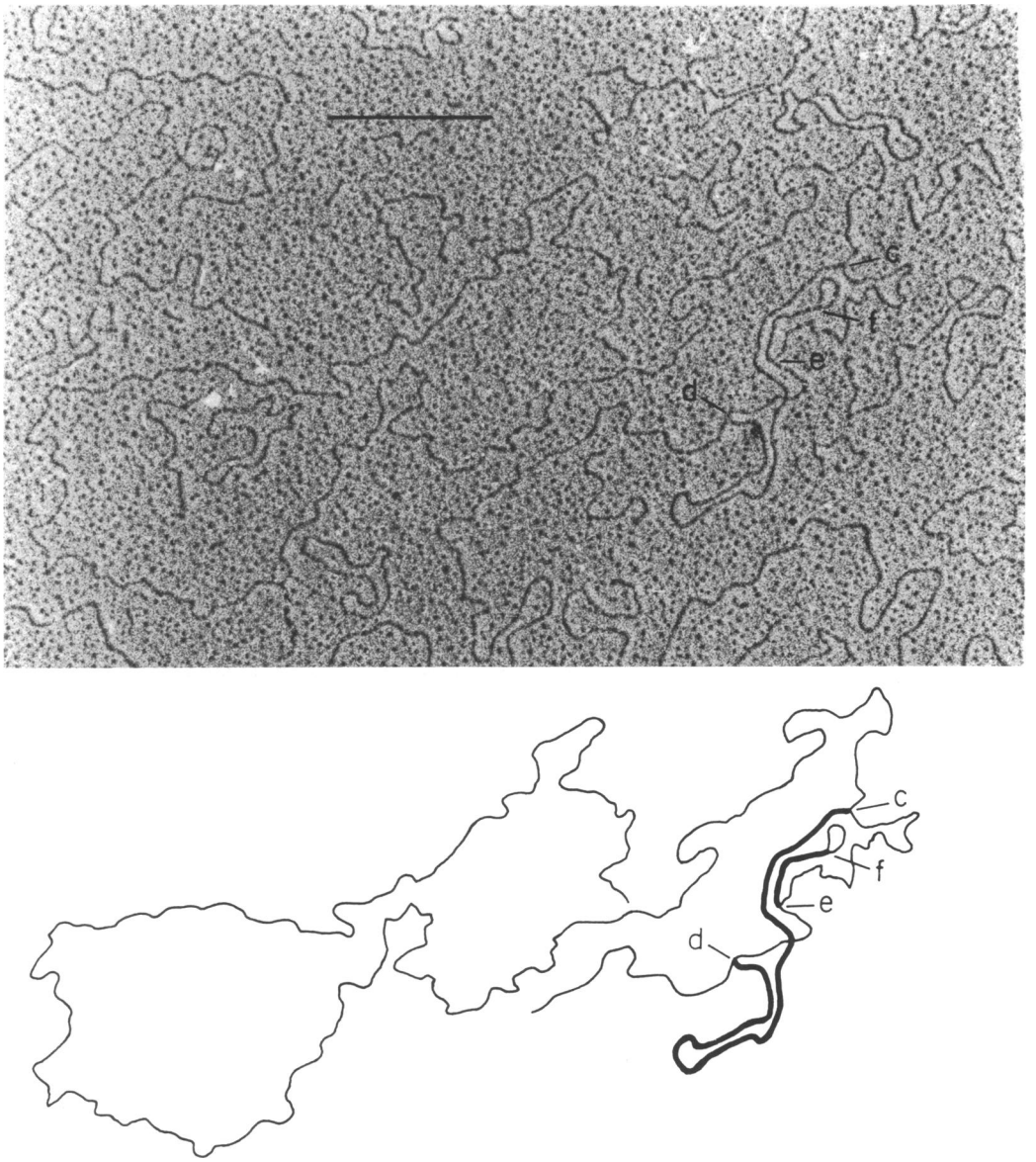

FIG. 4. *Electron micrograph of a heteroduplex between a circular single strand of pML31 and a broken strand of FΔ(33-43). A tracing of the heteroduplex is also shown. Arrows c and d denote the ends of the region of homology between pML31 and FΔ(33-43). Arrows e and f denote the ends of the inverted repeat of pML31. Bar represents a length of 0.5 μm.*

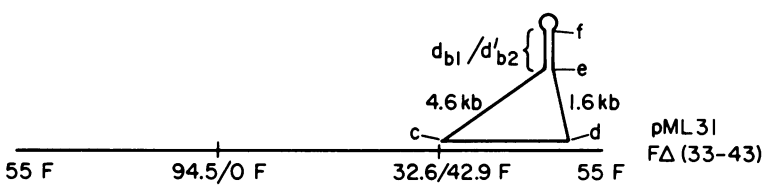

FIG. 5. *Diagram of the pML31/FΔ(33-43) heteroduplex.*

TABLE 3. *Length of inverted repeat sequences*

Feature	Molecular length (kb)	
	Heteroduplexes	Total molecules
IS2	1.3 ± 0.1 (12)[a]	1.3 ± 0.1 (25)[b]
Inverted repeat of pML31	1.0 ± 0.1 (20)	1.0 ± 0.1 (73)[c]
Ratio of inverted repeat of pML31/IS2	0.80	0.80
IS3		1.3 ± 0.1 (27)[d]
Inverted repeat of pML31		1.0 ± 0.1 (60)[e]
Ratio of inverted repeat of pML31/IS3		0.80

[a] The numbers in parentheses indicate the number of measurements.

[b] In addition to the 12 heteroduplexes, measurements of the IS2 stem of the inverted repeat from one single-stranded F8-33 circle and 12 broken F8-33 single strands were included.

[c] In addition to the 20 heteroduplexes, measurements of the inverted repeat from 20 single-stranded pML31 circles and 33 broken pML31 single-stranded molecules were included.

[d] The 27 measurements were all taken from broken R6 single-stranded fragments. The feature was identified as IS3 by two criteria. (i) The length of the single-stranded loop associated with the inverted repeat was 6.0 ± 0.5 kb, which agrees with the size of the loop associated with the IS3 inverted repeat of R6 (6.7 ± 0.3 kb; reference 15); (ii) on two fragments, two inverted repeats were observed; one was the size expected for IS3 and the other was the size expected for the d_{b1}/d'_{b2} inverted repeat (15). The distance between these two inverted repeats (23.9 ± 2.4 kb) was close to that expected for the separation of the two inverted repeats of R6 (20 kb).

[e] The 60 measurements include 14 intact pML31 circles. The length of the stem in these molecules was 1.0 ± 0.1 kb, the same as in the case of the total molecules. The remaining molecules were all broken single strands. Most of these were pML31 molecules, but some probably were R6 fragments, the inverted repeat being d_{b1}/d'_{b2}.

general agreement with the earlier evidence and, taken together, the two lines of evidence indicate that the genes responsible for the ability of F to maintain itself in the extrachromosomal state are located in the 6-kb region 42.9-49.3F.

The information available about the plasmid-specified gene products involved in the F replication system is minimal. The existence of plasmid mutations that render F maintenance thermosensitive (5, 10, 19) indicates that there is at least one F-coded protein required for plasmid maintenance. Since complementation analysis of such mutations is effectively prevented by incompatibility, the total number of F-coded proteins involved in plasmid maintenance is unknown. Beyond this, there is no proof that any of the properties that distinguish F replication from the replication of the main chromosome (sensitivity to acridine orange, incompatibility with other F-like replicons, thermosensitivity of wild-type F in certain hosts) require plasmid-encoded gene products. It should be noted that the involvement of host genes in F maintenance has clearly been demonstrated (5, 19, 24, 40) and host mutations that affect such properties as acridine orange sensitivity (24) and incompatibility (3, 34) have recently been described.

In addition to gene products, replication also appears to require a DNA site to act as an origin of replication. Although the existence of such an origin has not been directly demonstrated in the case of F, there is ample evidence for the existence of a unique origin of replication in many other systems (8). We assume, therefore, that F and pML31 each contain a unique origin of replication. Pritchard and his colleagues have presented a model that can account for the existence of more than one DNA sequence in a replicon which is capable of acting as an origin, even though only one is normally utilized (30, 31). In the present case, therefore, there are several possibilities for the relationship between the DNA sequence acting as origin on F and the DNA sequence acting as origin on pML31: (i) there is a unique origin sequence on F and the same origin sequence is used on pML31; (ii) there are several sequences on F capable of acting as an origin of replication, and the origin used on pML31 is not that one normally utilized on F; and (iii) there is no F sequence on pML31 that can function as an origin of replication, but such a sequence fortuitously is present on the *km* fragment. The finding that one of the two replication origins of the R factor NR1(28) is located in a region that may be homologous to the *km* fragment of pML31 suggests that there may be an origin sequence on *km*.

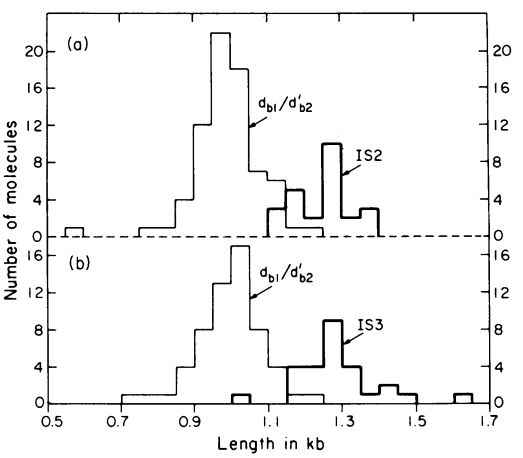

FIG. 6. *Histograms of the length distribution of the inverted repeat of pML31 compared with the length distribution of (a) the IS2 stem of the inverted repeat of F8-33 and (b) the IS3 stem of the inverted repeat of R6.*

However, recent studies on the replication of the pML31 plasmid in intact *Escherichia coli* cells clearly indicate that the origin of replication of pML31 is in the F fragment (R. Eichenlaub, D. Figurski and D. Helinski, manuscript in preparation). In addition, the *km* fragment in pML31 has been replaced by a fragment of DNA carrying several genes of the tryptophan operon (D. Figurski and D. Helinski, unpublished data). This mini-F *trp* hybrid plasmid exhibits replication properties similar to that of pML31.

The origin for conjugal DNA transfer (*oriT*) has been preliminarily mapped at or near 62.2F (M. Guyer, N. Davidson, and A. J. Clark, manuscript in preparation; A. J. Clark, N. Crisona, H. Nagaishi, and R. Skurray, *in Molecular Mechanisms in the Regulation of Gene Expression* [ICN Keystone Conference, March 1976], in press). Since our results show that the origin of vegetative replication (*oriV*), if unique, is located between 42.9 and 49.3F, it is unlikely that *oriT* and *oriV* coincide (12).

Timmis et al. (38) have also isolated a replication fragment from an F*lac* plasmid, using techniques similar to those employed in the isolation of pML31 (23). This hybrid plasmid, designated pSC138, has replication properties similar to those of pML31. Both plasmids are sensitive to acridine orange curing and each is incompatible with the parent F*lac* plasmid. Timmis et al. (38) reported that the F*lac*-derived region of pSC138 was 7.8 kb in length, relative to double-stranded PM2 DNA. After re-estimation of the length of PM2 DNA (22, 29; R. Skurray, M. Guyer, K. Timmis, F. Cabello, S. Cohen, N. Davidson, and A. J. Clark, J. Bacteriol., in press), this value was readjusted to 9.2 kb, in agreement with our results. Heteroduplex analysis has also demonstrated the identity of the F*lac*-derived components of pML31 and PSC138 (Skurray et al., in preparation).

The second *Eco*RI fragment of pML31 was originally obtained from R6-5 (R6-5 *Eco*RI fragment 5; 4). It contains the *km* gene(s) and an inverted duplication of a 1-kb sequence. The duplicated sequences are separated by 1 kb. A similar structure is found on the parent R6-5 plasmid (35) and on pML21, the ColE1-*km* chimera that was the immediate source of the fragment (23; see Fig. 1 of reference 17). Thus, the inverted duplication found on pML31 is not an artifact of the biochemical manipulations to which the R6-5 restriction fragment has been subjected but is a normal structural feature of this sequence.

In several recent cases, inverted, nontandem duplications have been found to be associated with plasmid genes controlling drug resistance. In the case of plasmid R6, a gene involved in the determination of resistance to tetracycline has been shown to be located in the region lying between the duplicated sequences (32, 35). In the case of plasmid RSF1030, which carries an *amp* gene specifying a TEM-β-lactamase, the ampicillin resistance has been shown to be able to translocate from one plasmid to another (11). A translocatable *amp* determinant has also been described by Kopecko and Cohen (21). In each case, the translocation of the drug resistance was found to be accompanied by the appearance of a nontandem, inverted repetition in the recipient plasmid. As no other sequences were translocated and since the duplicated sequences in each case were only 100 to 200 base pairs long, the *amp* genes must also be located in the region between the duplications. A translocatable *kan* sequence from plasmid JR67 has also been identified with an inverted repeat (2). By analogy, we suggest that the *km* gene(s) of pML31 and R6-5 is located between the inverted repeat sequences we have observed. We cannot, however, rule out the possibility that the coding sequence(s) determining kanamycin resistance lies elsewhere on the *Eco*RI fragment derived from R6-5, perhaps even in the repeated sequences.

The phenomenon of translocatable drug resistance has led to the hypothesis that the inverted repeat structure is mechanistically involved in the ability to translocate and that this process has played an important role in the evolution of R plasmids (2, 11, 20, 21, 32); it should be noted, however, that inverted duplications have not invariably been found to be associated with translocatable drug resistance (2, 9).

The recombinational mechanism of translocation is not known. The duplicated sequence associated with the *tet* gene of R6 has been shown to be homologous with insertion sequence IS3 (32). Insertion sequences also appear to be able to translocate (6, 13). For this reason, we were interested in determining whether the inverted repeat on pML31 could be associated with any of the known IS sequences. A direct comparison of their lengths showed that both IS2 and IS3 are longer than the repeated sequence of pML31. Similarly, the pML31 sequence cannot be identical to IS1 (which is only 700 to 800 base pairs in length; 13, 15). The pML31 sequence can also be distinguished from the inverted repeat associated with the *kan* gene of JR67. Berg et al. (2) have shown that the repeated sequence in the latter case is 1.5 kb in size and that the single-stranded loop between the repeats is 2.3 kb. These dimen-

sions are clearly different from those of the pML31 structure (Table 2). Thus, it appears on the basis of length measurements that the inverted repeat sequence of pML31 (and therefore R6-5) is not identical to any of the known IS sequences or inverted repeat sequences known to be associated with drug resistance genes on R plasmids. The possibility cannot be ruled out, however, that the inverted repeat of pML31 shares some sequence homology with any of these other sequences.

Finally, it should be noted that the *km* genes of both R6-5 and JR67 determine neomycin-kanamycin phosphotransferase activities (2, 35). This finding, and its implications for the evolution of R plasmids, cannot be analyzed without the information as to whether these enzymatic activities are determined by similar polypeptides. However, it is interesting to speculate that, if the polypeptides coded for by the two plasmids are similar and if the inverted repeats are different, then it is possible that a single nucleotide sequence can be associated with more than one set of inverted repeats. If, instead, the polypeptides determining the neomycin-kanamycin phosphotransferase activities are different and the two sets of inverted repeats turn out to share sequence relations, then it is possible for one inverted repeat to become associated with more than one coding sequence.

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